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Journal of Insect Science

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RESEARCH

Molecular Cloning and Xenobiotic Induction of Seven Novel Cytochrome P450 Monooxygenases in *Aedes albopictus*

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ABSTRACT. Cytochrome P450 monooxygenase (P450) is a superfamily of enzymes that is important in metabolism of endogenous and exogenous compounds. In insects, these enzymes confer resistance to insecticides through its metabolic activities. Members of P450 from family 6 in insects are known to play a role in such function. In this study, we have isolated seven novel family 6 P450 from *Aedes albopictus* (Skuse) (Diptera: Culicidae), a vector of dengue and chikungunya fever. Induction profile of these seven genes was studied using several insecticides and xenobiotics. It was found that deltamethrin and permethrin did not induce expression of any genes. Another insecticide, temephos, inhibited expression of *CYP6P15* for fivefold and twofold for *CYP6N29*, *CYP6Y7*, and *CYP6Z18*. In addition, copper II sulfate induced expression of *CYP6M17* and *CYP6N28* for up to sixfold. Benzothiazole (BZT), a tire leachate induced the expression of *CYP6M17* by fourfold, *CYP6N28* by sevenfold, but inhibited the expression of *CYP6P15* for threefold and *CYP6Y7* for twofold. Meanwhile, piperonyl butoxide (PBO) induced the expression *CYP6N28* (twofold), while it inhibited the expression of *CYP6P15* (fivefold) and *CYP6Y7* (twofold). Remarkably, all seven genes were induced two- to eightfold by acetone in larval stage, but not adult stage. Expression of *CYP6N28* was twofold higher, while expression of *CYP6P15* was 15-fold lower in adult than larva. The other five P450s were not differentially expressed between the larvae and adult. This finding showed that acetone can be a good inducer of P450 in *Ae. albopictus*. On the other hand, temephos can act as good suppressor of P450, which may affect its own bioefficacy because it needs to be bioactivated by P450. To the best of our knowledge, this is the first report on acetone-inducible P450 in insects. Further study is needed to characterize the mechanisms involved in acetone induction in P450.

Key Words: cytochrome P450 monooxygenase, induction, acetone

Metabolic resistance caused by cytochrome P450 monooxygenases (P450s) is the most common mechanism that confers insecticide resistance in insects (Scott 1999). P450 belongs to a superfamily of ubiquitous enzymes that can be found virtually in all eukaryotes and even in bacteria. This superfamily consists of more than 12,000 named isozymes, with over 2,000 of them belonging to insects. P450 is unique to each species and no organism shares identical P450 (Nelson 2011). The profile of P450 in a species shapes its individuality, interaction with abiotic and biotic factors, response to environmental and ecological changes, and ultimately the fitness of a species to survive and propagate (Nelson 1999, Ranson et al. 2002, Li et al. 2004).

P450 that has been implicated in conferring resistance in insect pests mostly consist of isozymes from family 6, which include *CYP6G1* and *CYP6A2* in *Drosophila melanogaster* Meigen (Daborn et al. 2002, Amichot et al. 2004), *CYP6CY3* in *Myzus persicae* Sulzer (Puinean et al. 2010), *CYP6P3* and *CYP6Z1* in *Anopheles gambiae* Giles (Chiu et al. 2008, Muller et al. 2008), and *CYP6BQ9* in *Tribolium castaneum* Herbst (Zhu et al. 2010). These P450s were either upregulated, amplified, or underwent gain-of-function mutation, which enable them to rapidly metabolize insecticides compared with their susceptible counterparts.

Expression of P450 is inducible by substrates that it metabolize (Feyereisen 1999). Induction of P450 overexpression confers rapid turnover of xenobiotics in the insects' habitat or host plant to ensure their survival. If the overexpressed P450 is able to metabolize insecticides, the insect host may survive from exposure to that insecticide. Preexposure to nonlethal dose of insecticides, or xenobiotics or both, which induce expression of detoxification enzymes, would increase the tolerance of insects not only to that inducer but also other xenobiotics, insecticides or both (Boyer et al. 2006, Poupardin et al. 2008). For instance, Suwanchaichinda and Brattsten (2001) showed that exposure to pentachlorophenol-induced expression of P450 and increased tolerance of *Aedes albopictus* (Skuse) (Diptera: Culicidae) toward carbaryl. Hence, screening of genes by induction study via quantitative polymerase chain reaction (PCR) or microarray can provide clues on the importance of a particular P450 (David et al. 2006; Bautista et al. 2007; Poupardin et al. 2010; Zhou et al. 2010a,b).

Whole-genome sequencing revealed that *Aedes. aegypti* L. and *An. gambiae* have 160 and 105 P450-coding genes, respectively (Ranson et al. 2002, Strode et al. 2008). Of 160 genes in *Ae. aegypti*, 44 belongs to P450. This indicates the relative importance of family 6 P450 in that species. Information on these P450 sequences allows investigation to ascertain their importance in insecticide resistance, either in expression studies in resistant strain, induction studies, in vitro expression, or comparative modeling to identify possible substrates (Dombrowski et al. 1998, Nikou et al. 2003, David et al. 2005, Chiu et al. 2008, McLaughlin et al. 2008, Muller et al. 2008, Komagata et al. 2010, Poupardin et al. 2010). However, P450 sequence information on the equally important disease vector, *Ae. albopictus*, is scarce.

Ae. albopictus which is also known as the Asian tiger mosquito originated from Asia. It has spread to many corners of the world primarily by trading of tires, and is expected to continue to disperse (Gratz 2004, Benedict et al. 2007). *Ae. albopictus* is able to transmit several important diseases such as dengue fever, chikungunya fever, and yellow fever (Boromisa et al. 1987, Gratz 2004, Pialoux et al. 2007). More than 2.5 billion people are at risk of dengue and dengue hemorrhagic fever, and of them, 1.3 billion live in southeast Asia region (WHO 2006).

Table 1. Primer sets for verification of putative full-length cytochrome P450 cDNA with respective annealing temperature, T_a

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	T_a (°C)
Cyp6by8	GCA TCA AAC AAT CAT GTC CG	TGA CTG TTT CTA TAA TGG GTC G	52
Cyp6m17	GGA CAT CTT TTT ACT AAT AAC CGC C	AGC TTT TCC ACC TTC AGC CA	58
Cyp6n28	ATG TTG CTT TTC CTG CTG TTG TCG G	CTA ATA CGA CTC ACT ATA GGG C	65
Cyp6n29	AGA TGA TTG CAC TGC TGC TAA T	AAC TTT ATC GTG ACA CTA CTG GGT AA	53
Cyp6p15	ATG TTA GCT TAT TTA TTG GCG GTG GT	TCT TAT CGT ACC GAA GAT AAT TCC CTC	56
Cyp6y7	ATG TGG TTG GTT TAC CTA GTG TG	TCA TAG TTT TTC CAC TCT CAA CCA G	56
Cyp6z18	ATG TTG ATC ATC TAC ACG GTC GC	CAT TAC TTC TTC CTG TTG GAA ATC T	54

Here, we report the isolation of full coding sequences of seven novel family 6 P450 from *Ae. albopictus* via rapid amplification of cDNA ends (RACE). Expression of these genes after exposure to several xenobiotics and insecticides was studied to elucidate the possible role of these genes in conferring resistance.

Materials and Methods

RNA Extraction and First-Strand cDNA Synthesis. Total RNA was extracted using conventional hot phenol extraction method (Jowett 1986). First-strand cDNA was synthesized using RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD) using 100 ng of extracted RNA.

Partial P450 Fragment Synthesis. Degenerate primers: forward P450F (5′-GARACIYTIMGNAARTAYCC-3′) and reverse P450R (5′-ATRCADATICKIGGNCCYTC-3′) based on motif of family 6 P450 were used to generate partial sequence of family 6 P450 fragments (Huang et al. 2008). PCR was carried out with 94°C initial denaturation for 3 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The reaction mixture (50 µl) contained 2 µl of first-strand cDNA, 7 µM of each primer, 0.2 mM dNTP (deoxyribonucleotide) mix, 2.5 U *Taq* DNA polymerase recombinant (Fermentas, Glen Burnie, MD), 1× *Taq* buffer with KCl, and 1.75 mM $MgCl_2$. Amplicons of ∼250 bp were purified and ligated into pTZ57R/T (Fermentas, Glen Burnie, MD) and cloned using TransformAid Bacterial Transformation Kit (Fermentas, Glen Burnie, MD). Plasmids purified from 10 white colonies were sent for sequencing. Sequences obtained were analyzed to verify the presence of P450 consensus region. Gene-specific primers for RACE based on these sequences were designed using Primer3Plus (Untergasser et al. 2007).

Rapid Amplification of cDNA Ends. RACE was conducted using SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA). Preparation of RACE-ready cDNA and PCR protocol was carried out following manufacturer's protocol. Amplicons were gel analyzed, purified, cloned, and sequenced as mentioned above.

Verification of P450 cDNA Sequence. Based on the sequences obtained from the RACEs, primer pairs (Table 1) that spanned 5′ and 3′ region of each sequence were designed to verify that the 5′ fragment and the 3′ fragment came from the same cDNA. High-fidelity *Pfu* DNA polymerase (Fermentas, Glen Burnie, MD) was used for this purpose. Reaction mixture consisted of 2.5 U *Pfu* polymerase, 5 µl 10× *Pfu* buffer with 20 mM $MgSO_4$, 2 µl 10 µM each primer, 1 µl 10 mM dNTP mix, 2 µl template cDNA, and 39 µl nuclease-free water was amplified at 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, respective annealing temperature for 30 s and 5 min at 72°C. Final extension was 10 min at 72°C. A-tailing of purified PCR product was conducted before TA. Positive clones were sent for sequencing.

Analysis of Sequence Data. Nucleotide sequence was translated using ExPASy Proteomic Server (Gasteiger et al. 2003). BLASTp was conducted using server on http://blast.uthsc.edu/. Phylogenetic analysis was conducted using MEGA4 by neighbor-joining method (Tamura et al. 2007), and inferred tree was evaluated with 10,000 bootstrap replicates.

Table 2. Primer sets for qRT-PCR generated by AlleleID 7

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
rpL8	TAT TCT TAG CGT TCC TTG T	ACC ATT ACA ATC AAC AAC AA
Cyp6by8	TGA AGC CGC TTG TGA TAT G	CGA GAA CAG GAA CAG ATG G
Cyp6m17	CTC GGT CAA CCA AGA TAT	GTA GTT CAA ATC GCC ATT
Cyp6n28	GGA TGT GTT TGT GAA GGA T	CCA TCG TCA CCA AAT GAG
Cyp6n29	GAA GGT GTA AAG TGG AAG A	AAT AAT CGG AAG CAT CAG T
Cyp6p15	CGG ATA TTC AGG AGA GG	ATA ACC AGG TCG TAT GT
Cyp6y7	AAG GTG GTC AAG GAT ACG	TTC TTC AAC TTC AAC AGC AA
Cyp6z18	TGA GTA TCG AAC AGT GTG	CAT TGT GAA GGT TAT GGT

Normalization was conducted using *Ae. albopictus* ribosomal protein L8 (*rpL8*; GenBank M99055.1).

Induction by Xenobiotics and Preparation of RNA. Induction was carried out on both larvae and adults using xenobiotics and insecticides from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Late third to early fourth instar larvae were exposed to the following insecticides or xenobiotics for 24 h in 250 ml of water: water only, $CuSO_4$ (1.2 ppm), benzothiazole (BZT; 14.2 ppm), temephos (0.01 ppm), piperonyl butoxide (PBO; 1 ppm), and acetone (0.4%; Merck kGaA, Darmstadt, Germany); 3- to 4-d-old female adults were applied with 0.1 µl of 0.27 ppm permethrin, 0.12 ppm deltamethrin, water, and 99.8% acetone. Concentration of insecticides was LC/LD_{25} (lethal dose or lethal concentration which causes 25% mortality of the test population) determined earlier, while the other treatments caused 0–5% mortality. Total RNA of survivors was immediately extracted 24 h posttreatment using Qiagen RNeasy Mini Kit (Hilden, Germany) using 30 mg of sample per extraction, followed by DNase treatment using DNase I (Fermentas, Glen Burnie, MD) according to manufacturer's instruction. RNA extracted was quantified by spectrophotometer and qualified by gel analysis.

Quantitative Reverse Transcription PCR. Primer pairs for target sequences were designed using AlleleID 7 (Table 2), which takes into consideration template secondary structure. Length of amplicon ranged from 80 to 95 for all target genes, and 199 for reference gene. Quantitative reverse transcription PCR (qRT-PCR) was conducted in iQ5 PCR machine (Bio-Rad, Hercules, CA) using KAPA SYBR FAST One-Step qRT-PCR Kit (KAPA Biosystem, Woburn, MA). Standard curve for each primer set was conducted by five RNA concentrations of 10× dilution factor and computed using Bio-Rad iQ5 software (Bio-Rad). Each reaction mixture consisted of 10 µl of 2× KAPA SYBR FAST qPCR Master Mix, 0.4 µl of 10 µM forward and reverse primer each, 0.4 µl of 50× KAPA RT Mix, and ∼15 ng RNA and nuclease-free water up to 20 µl. Cycling protocol was as follows: 42°C for 5 min for cDNA synthesis, 95°C for 2 min to inactivate reverse transcriptase, followed by 40 cycles of denaturation at 95°C for 3 s, annealing at 57°C for 20 s, and extension at 72°C for 30 s. Melt curve analysis of the products was conducted in the same cycler: heating to 95°C for 1 min, decrease to 55°C for another minute, followed by taking continuous fluorescence reading while increasing the temperature from 55 to 95°C in steps of 0.5 with 10 s at each step for 81 cycles. Three biological replicates were conducted for each treatment, each with three technical

Table 3. Percentage sequence identity among seven *A. albopictus* family 6 P450 genes

Sequence identity	*Cyp6by8*	*Cyp6m17*	*Cyp6n28*	*Cyp6n29*	*Cyp6p15*	*Cyp6y7*	*Cyp6z18*
Cyp6by8	100	46	46	46	43	50	36
Cyp6m17	46	100	49	48	47	47	38
Cyp6n28	46	49	100	56	46	46	33
Cyp6n29	46	48	56	100	46	45	36
Cyp6p15	43	47	46	46	100	47	37
Cyp6y7	50	47	46	45	47	100	34
Cyp6z18	36	39	33	36	37	34	100

replicates. Relative expression was calculated by using a formula that accounts for expression efficiency (Pfaffl 2001). Normalization was conducted using *Ae. albopictus* ribosomal protein L8 (GenBank M99055.1). Control sample of respective induction was used as a calibrator. For each primer sets, no-template control reaction was conducted. Data were analyzed using one-way Mann–Whitney *U*-test, with $P < 0.05$ and transcriptional ratio $-$SE >1.5 or $+$SE <0.67 considered as significantly overtranscribed or undertranscribed (Poupardin et al. 2010).

Results

Isolation of Novel P450 From Ae. albopictus. Seven family 6 P450 cDNAs were isolated in this study (GenBank JF681159–JF681165; Supp Table 1). BLASTp showed that *CYP6M17* shares 91% identity with *CYP6M6*; *CYP6Y7* shares 94% identity with *CYP6Y3*; *CYP6Z18* shares 85% identity to *CYP6Z7*; *CYP6P15* shares 89% identity to *CYP6P12*; *CYP6N28* shares 78% identity to *CYP6N11*; *CYP6N29* shares 86% identity to *CYP6N9*; and *CYP6BY8* shares 89% identity with *CYP6BY1* of *Ae. aegypti*. Identities among the seven sequences are shown in Table 3. These sequences code for ~500 amino acids (Supp Table 1), which is the typical length of insect P450 (Feyereisen 1999). Motifs that are unique to cytochrome family were found in all sequences obtained: WXXXR in helix C, EXXR in helix K, oxygen-binding region AGXXT in helix I, PXRF, and PFXXGXXXCXG, where the highly conserved cysteine serves as the fifth ligand to the heme iron (Nelson 1998, Feyereisen 1999, Werck-Reichhart and Feyereisen 2000, Matambo et al. 2010).

Inferred tree generated from neighbor-joining method (Saitou and Nei 1987) showed that *CYP6N28* was closely related to *CYP6N29*, with confidence value of 88 and 56% identity (Fig. 1). They are clustered in the same topology as *CYP6M* members, indicating that subfamily M and N were divided more recently. High confidence value indicates the stability of tree topology, but whether or not it represents the true tree is not known (Holmes 2003). *CYP6Z18* is closely related to *CYP6Z1* of *An. gambiae* which is capable of metabolizing DDT (dichlorodiphenyltrichloroethane) at 100% confidence level, while *CYP6P15* is closely related to pyrethroid-metabolizing *CYP6P* members (Nikou et al. 2003, Chiu et al. 2008, Muller et al. 2008). *CYP6Y7* and *CYP6BY8* are closely related to each other than to other P450 that were implicated in resistance, indicating that both are probably not involved in resistance.

Expression Study. Expression level of *CYP6P15* was significantly higher (16-fold) in larval than in adult stage, while that of *CYP6N28* was significantly higher in adult than in larval stage (Fig. 2). Expressions of the other five genes were not significantly different between adult and larval stage.

Significant induction of *CYP6M17* (fourfold) and *CYP6N28* (sevenfold) by BZT was observed, while inhibition was observed in both *CYP6P15* (threefold) and *CYP6Y7* (twofold; Fig. 3). Exposure to $CuSO_4$ in larvae significantly caused differential expression of *CYP6M17* and *CYP6N28* for sixfold. *CYP6N28* was also induced by PBO (twofold), which suppressed *CYP6P15* (fivefold) and *CYP6Y7* (twofold). Temephos, on other hand, suppressed the expression of four genes (*CYP6P15*, *CYP6N29*, *CYP6Y7*, and *CYP6Z18*) for two- to fivefold. Both deltamethrin and permethrin did not significantly induce expression of any genes. Finally, acetone induced expression of all P450 up to eightfold in larvae but not in adult (Fig. 4).

Discussion

In species such as *An. gambiae* and *Ae. Aegypti*, where the whole genome sequence is available, microarray of all P450 has been used to identify possible detoxification genes that are involved in the metabolism of insecticides and xenobiotics (David et al. 2005). However, for important pest species such as *Helicoverpa armigera* Hübner and *Culex quinquefasciatus* Say, where whole genome sequence is not available, isolation of P450 genes or construction of P450 cDNA library would be required before further examination of their function can be carried out (Kasai et al. 2000, Komagata et al. 2010, Zhou et al. 2010a). As mentioned earlier, most cases of P450-mediated insecticide resistance involve family 6 P450. Hence, this study was conducted to isolate P450 of family 6.

Besides direct biochemical roles in insecticide resistance, isolation of P450 allows characterization, which provides information on the complex environment in the body of a vector that harbors pathogens. Strategies based on that information can then be developed to augment the efforts to eliminate vector-borne diseases. In addition, acquisition of more P450 sequence information enables screening of specific target sites for development of vector or pests' *CYP*-specific inhibitor or both. Recently, insect-specific inhibitor of acetylcholinesterase had been screened by targeting cysteine residual that is only found in the active site of certain insects but not vertebrates (Polsinelli et al. 2010). Another example would be through RNA interference using double-stranded RNA in transgenic crops (Gordon and Waterhouse 2007), or even specific inhibitor that targets essential P450s that can affect the viability of an insect (Chung et al. 2009, Mito et al. 2011). Transgenic cotton plant with dsRNA designed to inhibit *CYP6Ae14* in cotton bollworm had effectively decreased the tolerance of bollworm toward toxic gossypol present in cotton (Mao et al. 2007). Indeed, the method of delivery for this type of inhibitor other than through transgenic crops is yet to be developed (Hemingway and Craig 2004, Mito et al. 2011), but we would expect it to be feasible in the future with the advances in technology.

Several studies have showed that xenobiotics are able to induce expression of P450 (Bautista et al. 2007; Poupardin et al. 2008, 2010). This may have an impact on toxicology study and insect pest control if the overexpressed P450 is able to metabolize insecticides. *CYP6BG1*, which is inducible by permethrin in *Plutella xylostella* L. (Bautista et al. 2007), has shown to reduce the permethrin resistance level when knockdown by RNAi (Bautista et al. 2009). Induction of larval P450 by the herbicide glyphosate and xenobiotics such as benzo[a]pyrene and BZT was shown to significantly increase tolerance of *Aedes* larvae to insecticides (Suwanchaichinda and Brattsten 2001, Riaz et al. 2009a). In *Papilio polyxenes* F., metabolism of xanthotoxin by *CYP6B1* and *CYP6B3* is enhanced following induction by xanthotoxin itself (Petersen et al. 2001). Because P450 of family 6 is commonly involved in insecticide resistance, our study focused on the induction profile of family 6 P450.

Induction is known to be both tissue- and sex specific, and is dependent on the duration of exposure, dose, strain, as well as route of

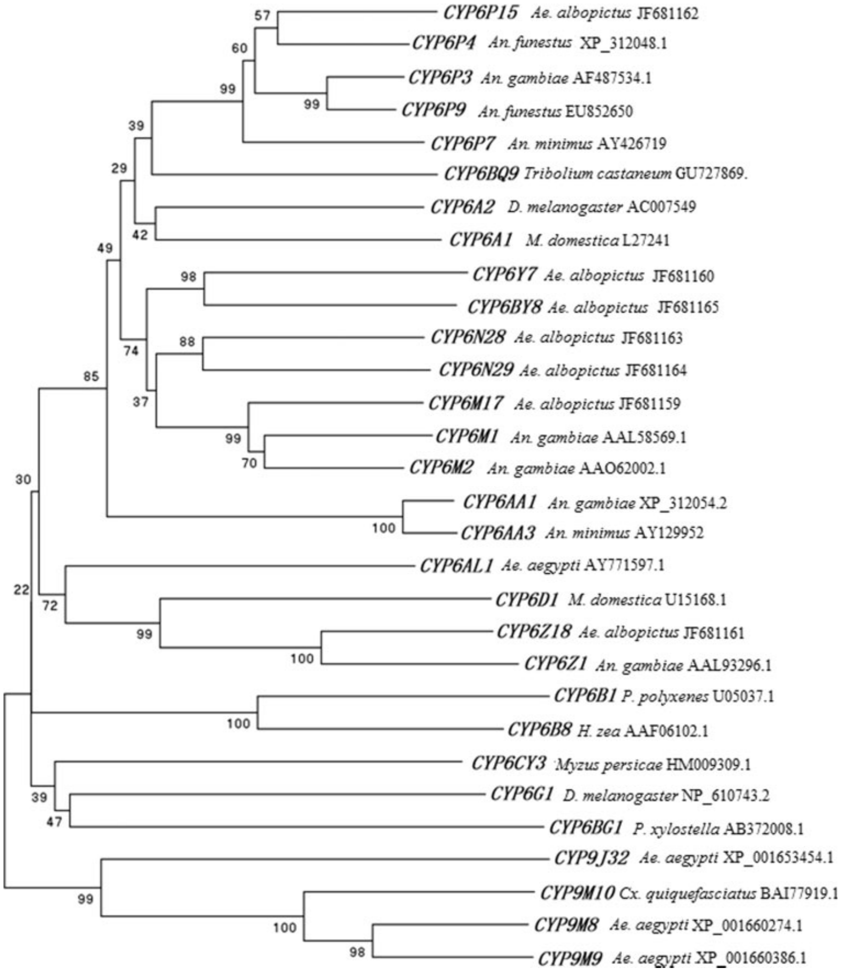

Fig. 1. Inferred tree generated by MEGA4 (Tamura et al. 2007) from sequences of isolated family 6 P450 together with selected P450 genes from Nelson (2009). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in <50% bootstrap replicates collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 30 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 296 positions in the final data set.

entry of inducer (Vontas et al. 2005, David et al. 2006, Le Goff et al. 2006, Bautista et al. 2007, Morra et al. 2010). Hence, induction studies using a xenobiotic should be conducted on life stage that would be likely to encounter it. In this study, both pyrethroids were tested on female adult instead of larvae as they are used more commonly as adulticides. However, other modifiers such as duration of exposure, dose, and strain used in this study most likely do not represent the actual condition in the field as these factors are not well understood and would be difficult to simulate.

In *Ae. albopictus*, BZT, a tire leachate, is known to induce peroxidation activity of tetramethybenzidine, and subsequently caused increased tolerance toward carbaryl, temephos, and rotenone. Intensity of P450 band was also increased after exposure to BZT, suggesting induction activity caused by BZT (Suwanchaichinda and Brattsten 2002). Because whole microsome was tested in that work, increased activity and amount of P450 should be caused by overexpression of multiple genes. But in this study, we identified significant induction of only *CYP6M17* and *CYP6N28* by BZT, while *CYP6P15* and *CYP6Y7* were undertranscribed.

Copper is one of the common metals that can be found in larval habitat (Mireji et al. 2008). Exposure of aquatic insects to heavy metals may induce increased tolerance toward the metal. The factor that causes this tolerance may also increase the tolerance toward insecticide. Poupardin et al. (2008) demonstrated that exposure of *Ae. aegypti* larvae to $CuSO_4$ increased both P450 activities and tolerance to permethrin and temephos. It was also demonstrated that $CuSO_4$ increased the expression of *CYP6M11* and *CYP6N12* (Poupardin et al. 2010). Interestingly, *CYP6M17* and *CYP6N28* were induced by $CuSO_4$ in this study. Perhaps, this is due to conserved induction mechanism shared by these closely related alleles. However, *CYP6N29* was not induced by $CuSO_4$ though.

From our experiment, deltamethrin and permethrin did not significantly induce expression of any selected *CYP*. Previous studies indicated that insecticides might not be a good inducer of P450 compared with other xenobiotics (Qiu et al. 2003, Vontas et al. 2005, Willoughby et al. 2006, Pridgeon et al. 2009, Riaz et al. 2009b, Lertkiatmongkol et al. 2010). Negative result in this study may be due to the fact that we examined whole-body gene expression rather than tissue-specific expression, as overexpression in specific tissues might be masked by the whole-body expression. Although induction by insecticides and subsequent increased tolerance to insecticides have been reported in

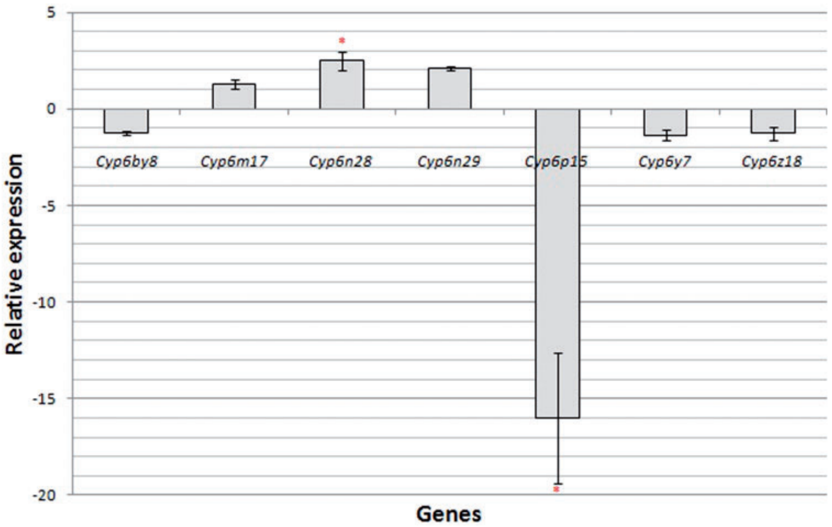

Fig. 2. Expression level of seven family 6 P450 genes in female adult relative to larva. Positive value indicates higher expression in adult than larva and vice versa. Error bars represent SE; asterisks represent significant difference in expression level ($P < 0.05$).

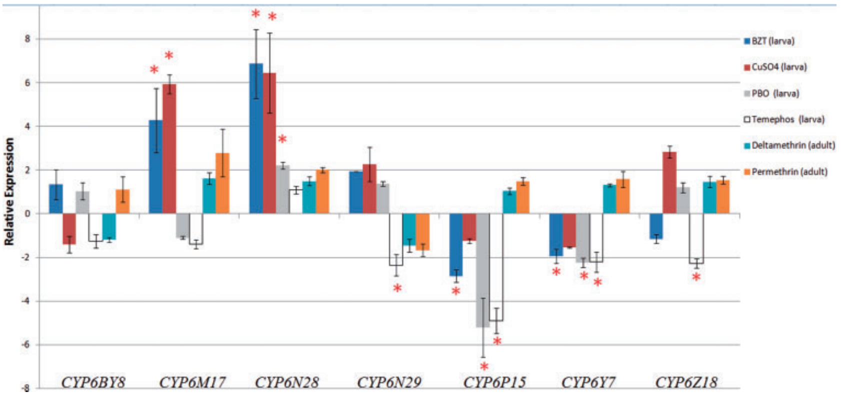

Fig. 3. Expression ratio of seven P450 after induction by BZT, $CuSO_4$, temephos, deltamethrin, and permethrin in relation to its respective calibrator. Positive value indicates overexpression, while negative value indicates underexpression. Error bars represent SE; asterisk indicates statistically significant differential expression ($P < 0.05$).

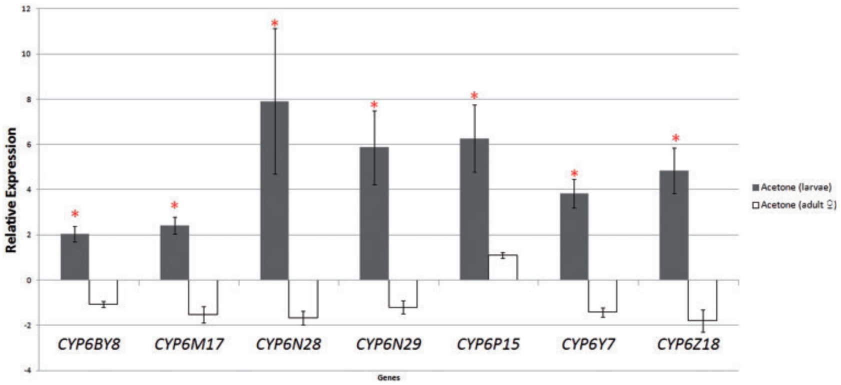

Fig. 4. Mean expression ± SE of family 6 P450 genes in *Ae. albopictus* after induction by acetone. Positive value indicates overexpression and negative value indicates otherwise. Error bars represent SE; asterisks represent significant differential expression ($P < 0.05$). Relative expressions of both larvae and adult are incomparable because calibrator samples used were different.

insects such as *P. xylostella* (Bautista et al. 2007) and *H. armigera* (Zhou et al. 2010a), Willoughby et al. (2006) suggested that induction of insecticide-metabolizing P450 by insecticide is not significant for the survival of insects. In their work, exposure of *D. melanogaster* to DDT induces minimal overexpression of detoxification enzyme, while the other five insecticides did not induce overexpression of any *CYP*. Giraudo et al. (2010) also suggested that relationship between induction and resistance is not significant. Although *CYP6P3* in *An. gambiae* is able to metabolize permethrin (Muller et al. 2008), it is not induced by permethrin (Vontas et al. 2005). However, it is also possible that the target P450 that we have selected is not involved in the metabolism of insecticides tested because only seven family 6 P450s were selected, whereas there are around 44 members in family 6 in *Ae. aegypti* (Strode et al. 2008), and more family 6 P450 genes in *Ae. albopictus* are expected.

In contrast, temephos suppressed the expression in four out of seven genes. Temephos is bioactivated in insects by P450 (Scott 1999). If transcription of bioactivation-related P450 is hindered, it may reduce the efficacy of temephos. Perhaps this is one of the reasons that temephos (or organophosphate) is a weaker insecticide than pyrethroids. This also indicates that P450 may be able to respond to protect its host from proinsecticides that need to be bioactivated through undertranscription. However, Poupardin et al. (2008) showed that preexposure to temephos did not increase or decrease the tolerance of mosquito larvae to temephos. It is also possible that the suppression of P450 by temephos causes lesser P450 available to detoxify bioactivated temephos-oxon, hence increasing its bioefficacy. This may compensate reduced bioefficacy caused by reduced bioactivation due to undertranscription of P450. Further experiment is required to confirm this.

The most striking outcome of our study is that acetone is a good inducer of *CYP*. Induction of some P450 such as *CYP2E1* by acetone was identified in vertebrates (Sinclair et al. 1990, Ronis et al. 1998, Gonzalez-Jasso et al. 2003); but to our knowledge, this was not demonstrated in insects, at least not in *Blatella germanica* L., which is the only work that we could find on insect (Brown et al. 2003). Pridgeon et al. (2009) suggested that acetone may induce expression of P450 as they were not able to identify overexpressed P450 after induction by permethrin through subtractive hybridization. If P450 is inducible by acetone, induction study using acetone as solvent for interested compounds may be hard to be interpreted because inductions by both acetone and target compound may be more than additive. Besides that, intense induction by acetone may also mask induction by compounds studied. Induction of P450 by acetone is observed only in larvae but not in adults, probably due to the difference in life stage treated. In *D. melanogaster*, different P450s were overexpressed when inducted using phenobarbital at the adult and larval stage (Willoughby et al. 2006). In addition, because acetone was applied on the pronotum of adult mosquito, it would evaporate quickly before it could induce the expression of any genes. It was known that induction can be both tissue- and sex specific, and is dependent on the duration of exposure, dose, strain, as well as route of entry of inducer (Vontas et al. 2005, David et al. 2006, Le Goff et al. 2006, Bautista et al. 2007, Morra et al. 2010).

Mechanism and consequences of induction seemed to be complicated and may vary according to inducer, gene, and strain. In *D. melanogaster*, a factor in 0.2–0.8 kb upstream of *CYP6A8* caused increased luciferase activity when induced by caffeine, DDT, and phenobarbital. However, similar *cis*-regulating factor is not observed in *CYP6G1* (Morra et al. 2010). Several inducers of *CYP2E1*, such as ethanol, are found to maintain the stability of CYP2E1 enzymes from degradation upon binding with the enzymes, thus increasing the level of CYP2E1 in the cell (Eliasson et al. 1988, Zanelli et al. 2000). Further study showed that a chaperon protein, HSP90, is involved in the dissociation of CYP2E1 from the membrane that allows CYP2E1 to be transferred for proteolysis in proteasome (Goasduff and Cederbaum 2000). Kitam et al. (2012) showed that interaction between ethanol and CYP2E1 causes large positive charges in the entrance to the active site and changes in protein surface charge of CYP2E1. This causes reduced interaction efficiency between HSP90 and CYP2E1. Consequently, the content of CYP2E1 increases. The same mechanism may be present in the insect P450. Even though organic solvents like acetone can induce expression of P450 such as *CYP2E1*, activities of induced P450 may be inhibited (Li et al. 2010). Perhaps it is an important strategy for an enzyme to be overexpressed upon contact with the inhibitor of enzyme to minimize the effect of reduced enzyme activity. Further study needs to be conducted to verify the mechanism of induction by acetone.

In this study, we isolated seven family 6 P450 from *Ae. albopictus*. Certainly, more P450 can be isolated if a more thorough study was conducted. This is a work planned for the next stage. Induction study showed that both *CYP6M17* and *CYP6N28* were highly inducible by xenobiotics compared with the rest. This suggests that these two genes are probably important in chemoprotective role. Furthermore, it was found that acetone was able to induce overexpression of all seven genes studied. From our search on Scopus database, this has not been reported in insect before. Mechanism of induction by acetone in insect P450 is not clear, and further works are required for a better understanding of it. We hope that the sequences obtained can serve as a basis for further characterization of *Ae. albopictus CYP6*s, as well as a better understanding of evolution using P450 as model.

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